Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants

Ilka Schoenian[a], Michael Spiteller[b], Manoj Ghaste[b], Rainer Wirth[c], Hubert Herz[d], and Dieter Spiteller[a,1]

[a]Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, D-07745 Jena, Germany; [b]Institute of Environmental Research of the Faculty of Chemistry, Dortmund University of Technology, D-44221 Dortmund, Germany; [c]Department of Plant Ecology and Systematics, Technical University of Kaiserslautern, D-67663 Kaiserslautern, Germany; and [d]Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancón, Republic of Panamá

Edited* by Jerrold Meinwald, Cornell University, Ithaca, NY, and approved November 22, 2010 (received for review June 16, 2010)

Leaf-cutting ants cultivate the fungus *Leucoagaricus gongylophorus*, which serves as a major food source. This symbiosis is threatened by microbial pathogens that can severely infect *L. gongylophorus*. Microbial symbionts of leaf-cutting ants, mainly *Pseudonocardia* and *Streptomyces*, support the ants in defending their fungus gardens against infections by supplying antimicrobial and antifungal compounds. The ecological role of microorganisms in the nests of leaf-cutting ants can only be addressed in detail if their secondary metabolites are known. Here, we use an approach for the rapid identification of established bioactive compounds from microorganisms in ecological contexts by combining phylogenetic data, database searches, and liquid chromatography electrospray ionisation high resolution mass spectrometry (LC-ESI-HR-MS) screening. Antimycins A_1–A_4, valinomycins, and actinomycins were identified in this manner from *Streptomyces* symbionts of leaf-cutting ants. Matrix-assisted laser desorption ionization (MALDI) imaging revealed the distribution of valinomycin directly on the integument of *Acromyrmex echinatior* workers. Valinomycins and actinomycins were also directly identified in samples from the waste of *A. echinatior* and *A. niger* leaf-cutting ants, suggesting that the compounds exert their antimicrobial and antifungal potential in the nests of leaf-cutting ants. Strong synergistic effects of the secondary metabolites produced by ant-associated *Streptomyces* were observed in the agar diffusion assay against *Escovopsis weberi*. Actinomycins strongly inhibit soil bacteria as well as other *Streptomyces* and *Pseudonocardia* symbionts. The antifungal antimycins are not only active against pathogenic fungi but also the garden fungus *L. gongylophorus* itself. In conclusion, secondary metabolites of microbial symbionts of leaf-cutting ants contribute to shaping the microbial communities within the nests of leaf-cutting ants.

chemical imaging | antibiotics | chemical defense | ecological function

Leaf-cutting/fungus-growing ants such as *Acromyrmex* are unique among ants, because they grow the fungus *Leucoagaricus gongylophorus* (Agaricales: Leucocoprineae) with harvested leaf material in chambers of their nests (1). In turn, *L. gongylophorus* is their major food source. However, this obligate mutualistic interaction is threatened by various microbial pathogens such as the fungi *Escovopsis* (2, 3), *Fusarium* (4), *Syncephalastrum* (4), and *Trichoderma* (4). In addition, microorganisms from the surrounding soil or plant pathogens accidentally introduced from harvested leaf material may compete with the garden fungus for nutrients and living space (5). Therefore, leaf-cutting ants treat their fungus gardens with great care, removing any suspicious material into waste chambers (6). Besides this mechanical cleaning behavior, leaf-cutting ants make use of antimicrobial chemicals (7–9); these include 3-hydroxydecanoic acid, which is secreted from the ants' metapleural glands.

However, in 1999, Currie et al. (10) discovered microbial symbionts, identified as *Pseudonocardia*, from biofilms on the integument of leaf-cutting ants. Because *Pseudonocardia* belong to the well-known antibiotic-producing *Actinobacteria*, it was suspected that *Pseudonocardia* play a crucial role in the ants' defense against pathogens. Although isolated microbial symbionts were active against *Escovopsis* in the agar diffusion assay (10), until recently, not a single compound from the ants' microbial symbionts had been characterized. Using bioassay-guided isolation, Haeder et al. (11) identified the antifungal candicidin macrolides that are produced by a large number of *Streptomyces* symbionts isolated from three different leaf-cutting ant species (*A. octospinosus*, *A. echinatior*, and *A. volcanus*). For the fungus-growing ant *Apterostigma dentigerum*, Oh et al. (12) reported the cyclodepsipeptide dentigerumycin from a *Pseudonocardia* symbiont with activity against *E. weberi*. From *A. octospinosus*, Barke et al. (13) detected, besides the presence of the previously characterized candicidin polyene macrolide-producing *Streptomyces* (11), a *Pseudonocardia* symbiont that produces a nystatin-like polyene macrolide. These recent findings indicated that there are likely to be a number of diverse antifungal compounds yet to be identified from microbial symbionts of leaf-cutting/fungus-growing ants (11–13).

Over the last few years, researchers have realized that the ecosystem of leaf-cutting ants is much more complex than initially described as a coevolution of the leaf-cutting ants, their fungus garden *L. gongylophorus*, one microbial symbiont (*Pseudonocardia*), and one specialized fungal pathogen (*Escovopsis*) (14). In addition to the characterization of microbial antifungal compounds (11–13) involved in mediating the interactions between the different partners, it has become evident that both a large number of pathogens (2–5) pose a threat to the ants' fungus garden and a large diversity of microbial symbionts can be found within the ants' nests. These symbionts fulfill diverse functions, including defense against pathogens or promotion of the growth of the garden fungus (e.g., by nitrogen fixation) (10–13, 15–17). Because some bacterial symbionts of leaf-cutting ants can be also detrimental to the growth of the mutualistic fungus *L. gongylophorus*, the one-sided view of leaf-cutting ants and *Actinomyces* symbionts as mutualistic partners of the leaf-cutting ants should be challenged (18).

In order to begin to better understand the ecological role of microorganisms associated with leaf-cutting ants, it is crucial to expose the chemistry of the individual (micro)organisms in the community. Extending phylogenetic comparisons of secondary metabolite producers (19–21), we used a combination of phylogenetic analysis, database screening, and electrospray ionisation high resolution mass spectrometry (ESI-HR-MS) analysis to

Author contributions: I.S. and D.S. designed research; I.S., M.S., M.G., R.W., H.H., and D.S. performed research; I.S. and D.S. analyzed data; and I.S. and D.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. HM538453 and HM538454).

[1]To whom correspondence should be addressed. E-mail: dspiteller@ice.mpg.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008441108/-/DCSupplemental.

rapidly identify ecologically relevant secondary metabolites from microbial symbionts. In this way, we identified several antibiotics and investigated their role in shaping the complex interactions in the leaf-cutting ants' ecosystem.

Results

Structure Elucidation of Antimicrobial and Antifungal Compounds from Microbial Symbionts. A variety of microbial symbionts from three different *Acromyrmex* leaf-cutting ant species (*A. octospinosus*, *A. echinatior*, and *A. volcanus*) had been isolated in a previous study. The symbiotic microorganisms were characterized using their 16*S* rDNA sequences for database comparison (11). In addition, many more 16*S* rDNA sequences from microbial symbionts of leaf-cutting ants are now available (11, 16, 22–24). However, because most research concerning the symbionts of leaf-cutting ants has focused on *Pseudonocardia*, their 16*S* rDNA sequences are the main ones that have been collected and used to study the evolution of the symbiosis between *Pseudonocardia* and leaf-cutting ants (14, 23).

Instead of using the 16*S* rDNA sequences for evolutionary studies (19–21), we used phylogenetic data as a guideline to rapidly identify secondary metabolites that might play a crucial role in the interactions of the complex microbial community of leaf-cutting ants. We identified the closest well-studied relatives to *Streptomyces* symbionts from leaf-cutting ants based on their 16*S* rDNA sequence similarity to sequences from the Greengenes and National Center for Biotechnology Information (NCBI) databases using the blast algorithm. The secondary metabolite production of the identified relatives was then studied using the Chemical Abstracts Service (CAS) SciFinder database. On the basis of the results from this phylogenetic comparison, culture supernatants and methanol extracts of the microbial symbionts from *Acromyrmex* ants were screened by liquid chromatgraphy mass spectrometry (LC-MS) in a search for the $[M+H]^+$ ions of suspected natural products. Putative hits were verified by comparing the retention times and ESI-HR-MS spectra of selected compounds with those of authentic standards.

For example, *Streptomyces* sp. Av25_2 showed high similarity to *Streptomyces parvus* str. NBRC 14599 (AB184603.1; 99.57%) (Table S1 and Fig. S1). Another *S. parvus* strain had previously been characterized as a producer of actinomycin C (25). In light of this information, we used LC-MS to screen *Streptomyces* sp. Av25_2 for actinomycin production. Indeed, the symbiotic strain *Streptomyces* sp. Av25_2 was found to produce actinomycin D (**1**) and the closely related actinomycin X_2 (**2**) (26, 27) (Fig. 1, Table S1, and Fig. S1). The identity of actinomycin D (**1**) and X_2 (**2**) was further confirmed by NMR and comparison with commercially available actinomycin standards. Valinomycin (**3**) (28) and its closely related derivatives (29) were identified from the symbiont *Streptomyces* sp. Av25_3, revealing its high similarity to valinomycin producers (Fig. 1, Table S1, and Fig. S1) such as *S. anulatus* (EU647474.1; 98.84%) (Table S1 and Fig. S1) and *S. tsusimaensis* (EU622279; 99.21%) (Table S1 and Fig. S1) (20). Furthermore, we identified the well-known antifungal antimycins A_1–A_4 (**4–7**) (30) (Fig. 1, Table S1, and Fig. S1), which are produced by microbial symbionts of *Acromyrmex*, on the basis of the high similarity of *Streptomyces* sp. Ao10 to *S. albidoflavus* (DQ855477.1; 99.72%) (Table S1 and Fig. S1). *S. albidoflavus*, which was recently isolated from mangroves, has been shown to produce antimycin A_{18} (31), suggesting the possible production of antimycins (**4–7**) by *Streptomyces* Ao10.

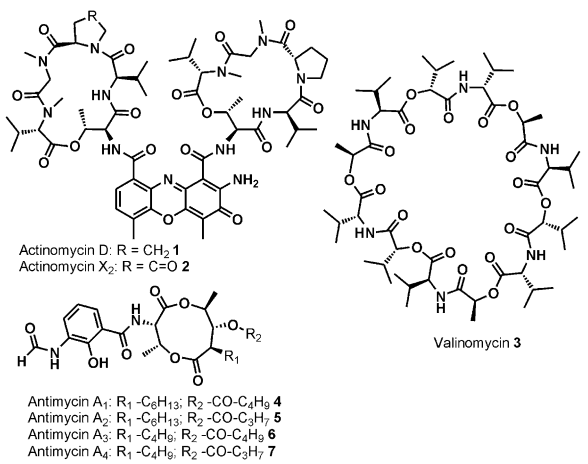

Fig. 1. Structures of actinomycin D (**1**), actinomycin X_2 (**2**), valinomycin (**3**), and antimycins A_1–A_4 (**4–7**) produced by *Streptomyces* associated with leaf-cutting ants.

The LC-MS screening of the extracts from all *Streptomyces* isolates (11) from leaf-cutting ants revealed the distribution of the identified antibiotics in the microbial isolates from three *Acromyrmex* species (*A. octospinosus*, *A. volcanus*, *A. echinatior*). Valinomycins were produced by *Streptomyces* sp. Av25_3, *Streptomyces* sp. Av26_3, and *Streptomyces* sp. Av25_6. Antimycins seem to be widespread among *Streptomyces* and were found in one-half of the *Streptomyces* symbionts analyzed, whereas actinomycins were only produced by *Streptomyces* sp. Av25_2 (Table S1).

Function of Secondary Metabolites from the Symbionts of Leaf-Cutting Ants. To evaluate the ecological role of the identified secondary metabolites from the microbial symbionts of *Acromyrmex* leaf-cutting ants, we tested the compounds in agar diffusion assays against fungal pathogens of the fungus garden (*E. weberi*, *F. decemcellulare*, and *T. harzianum*) and insect pathogenic fungi (*Metarhizium anisopliae*, *Beauveria bassiana*, and *Cordyceps militaris*). The black yeast *Phialophora fastigiata* (32) and the fungus *Syncephalastrum racemosum* (4), both of which are known pathogens in the nests of leaf-cutting ants, were included in the agar diffusion assays as well. We also examined the inhibitory potential of actinomycins (**1**, **2**), valinomycin (**3**), valinomycin derivatives, and antimycins (**4–7**) against the common soil bacterium *Bacillus subtilis*. In addition, the effects of the bioactive substances identified (**1–7**) were also investigated (Fig. 1) on selected nonproducing microbial isolates from *Acromyrmex* leaf-cutting ants and the mutualistic fungus *L. gongylophorus*. The results of the bioassays are presented in Table 1, Table S2, and Figs. S2–S8.

Actinomycin D (**1**) strongly inhibited *B. subtilis* (Fig. S5) as well as the growth of *Streptomyces* sp. Av25_4 (Fig. S6) but not *Streptomyces* sp. Ao10 associated with the leaf-cutting ants (Fig. S6). Even as little as 0.4 nmol actinomycins (**1**, **2**) hampered the growth of *Pseudonocardia* sp. Ao1 and *Pseudonocardia* sp. Av30 (Fig. S6) in the agar diffusion assay. Greater quantities of actinomycins also inhibited the growth of the fungi *F. decemcellulare*, *S. racemosum*, and the black yeast *P. fastigiata* (Fig. S4).

The well-known antifungal antimycins (58 nmol) (**4–7**) created inhibition zones in the agar diffusion assay against *E. weberi* (2, 3) (Fig. S2), the black yeast *P. fastigiata* (32) (Fig. S4), and *C. militaris* but not against other insect pathogenic fungi (e.g., *B. bassiana*) tested.

As little as 5.8 nmol antimycins A_1–A_4 (**4–7**) clearly inhibited the growth of the leaf-cutting ants' mutualistic fungus *L. gongylophorus* in the agar diffusion assay. In contrast, valinomycins (up to 240 nmol) did not inhibit the growth of *L. gongylophorus* (Fig. S3). In our bioassays, valinomycins inhibited only the growth of *B. subtilis* (Fig. S5).

Mixtures of actinomycins (**1**, **2**), valinomycin (**3**), valinomycin derivatives, antimycins (**4–7**), and candicidins (11) exhibit strong synergistic effects (33, 34). Combined with valinomycins or antimycins (**4–7**), candicidin macrolides close to or below the minimal inhibitory concentration (MIC, around 1–6 nmol each) caused

Table 1. Inhibition zones caused by antimycins A_1–A_4 (4–7), actinomycin D (1) and X_2 (2), and valinomycin (3) and valinomycin derivatives in the agar diffusion assay against fungi and bacteria

Organism	Antimycins (nmol/10 mL SFM cm inhibition zone)	Actinomycins (nmol/10 mL SFM cm inhibition zone)	Valinomycins (nmol/10 mL SFM cm inhibition zone)
Leucoagaricus gongylophorus	5.8/58	2.4/24	2.7/27
	1.0/1.7	X/0.2	X/X
Escovopsis weberi	5.8/58	2.4/24	2.7/27
	0.3/0.4	X/X	X/X
Phialophora fastigiata	58/280	1.2/240	2.7/270
	0.1/0.2	X/0.2	X/X
Trichoderma harzianum	58	24	27
	0.3	X	X
Beauveria bassiana	27.5	4/24	1.4/270
	X	X/X	X/X
Metarhizium anisopliae	27.5/58	4/24	1.4/270
	X/X	X/X	X/X
Cordyceps militaris	27.5/58	4/24	1.4/270
	0.1/0.2	X/X	X/X
Fusarium decemcellulare	580	240	270
	X	0.1	X
Streptomyces sp. Av 25_4	27.5	4/12	1.4
	X	0.2/0.8	X
Streptomyces sp. Av 25_2	27.5	12	—
	X	X	—
Streptomyces sp. Ao10	27.5	4	1.4
	X	X	X
Pseudonocardia sp. Ao1	27.5	4/12	1.4
	X	0.1/0.8	X
Pseudonocardia sp. Av30	27.5	0.4/12	1.4/2.7
	X	0.1/0.6	X/X
Bacillus subtilis	275	0.4/2/12	270
	X	0.3/0.8/1.1	0.2
Syncephalastrum racemosum	145	120	120
	X	0.1	X

SFM, soy flour medium; X, no inhibition; —, not tested.

clear inhibition zones in the agar diffusion assay against *E. weberi* (Table S2 and Figs. S7 and S8).

Direct Screening for Antimicrobial Compounds in the Fungus Garden, in the Waste, and on the Integument of Leaf-Cutting Ants. To more directly assess the ecological role of secondary metabolites identified from the microbial symbionts of leaf-cutting ants, we used LC-MS to identify these in extracts of the fungus garden and waste material. Fungus garden samples (0.58–1.40 g, fresh weight) and waste material (0.58–31.81 g, fresh weight) from *A. niger* and *A. echinatior* were collected. The samples were extracted with ethyl acetate. The extracts were concentrated, redissolved in methanol, and subjected to LC-MS analysis. Actinomycin X_2 (**2**) was detected only in a few waste samples from *A. echinatior*. In contrast, valinomycins were found in 10 of 13 samples. An external calibration curve was used to estimate the amounts of valinomycins and actinomycins present in the waste of leaf-cutting ants. Actinomycins were found at concentrations of the order of 170 pmol/g. The quantity of valinomycins in the various waste samples varied considerably, ranging from 0 to 13 nmol/g. Actinomycins (**1**, **2**) and valinomycins were not detected in fungus garden samples. In the limited sample material available to us, we were unable to directly detect antimycins A_1–A_4 (**4–7**) in waste or fungus garden samples.

Valinomycin (**3**) was detected directly on the integument of *A. echinatior* workers using matrix-assisted laser desorption ionization (MALDI) imaging. Valinomycin (**3**) was found in varying concentrations and at various positions on the ants' bodies. For some *A. echinatior* workers, valinomycin (**3**) was not only detected on the body, particularly, the alitrunk, but also on the legs. Judging from the MALDI imaging, *A. echinatior* ants can have several nanograms of valinomycin (**3**) on their cuticle. It is noteworthy that valinomycin (**3**) seems to be often produced in highest amounts in highly localized patches (Fig. 2).

Discussion

Phylogenetic analysis is widely used to classify microorganisms and more recently, to study evolutionary relationships among secondary metabolite producers (19–21). Here, we show that 16*S* rDNA data combined with database search and LC-MS profiling can be highly valuable for quickly identifying secondary metabolite profiles from new microbial isolates. This straightforward method has great potential to help reveal the chemistry in various complex microbial associations.

Using the 16*S* rDNA analysis combined with database searching and LC-MS screening, we have identified valinomycins, actinomycin D (**1**), actinomycin X_2 (**2**), and antimycins A_1–A_4 (**4–7**) (Fig. 1, Table S1, and Fig. S1) as compounds that are produced by microbial symbionts of *Acromyrmex* ants. Actinomycins (**1, 2**) were found only from a single *A. volcanus* symbiont *Streptomyces* sp. Av25_2, whereas valinomycins and antimycins A_1–A_4 (**4–7**) were produced by several of the *Streptomyces* symbionts analyzed. Similarly, candicidin macrolides were synthesized by the majority of the *Streptomyces* isolates from the three *Acromyrmex* species tested (11). The broad occurrence of those antibiotics suggests their important role in the ecosystem of the *Acromyrmex* ants.

The antifungal antimycins A_1–A_4 (**4–7**) inhibit the electron transfer of the ubiquinol:cytochrome *c* reductase (complex III)

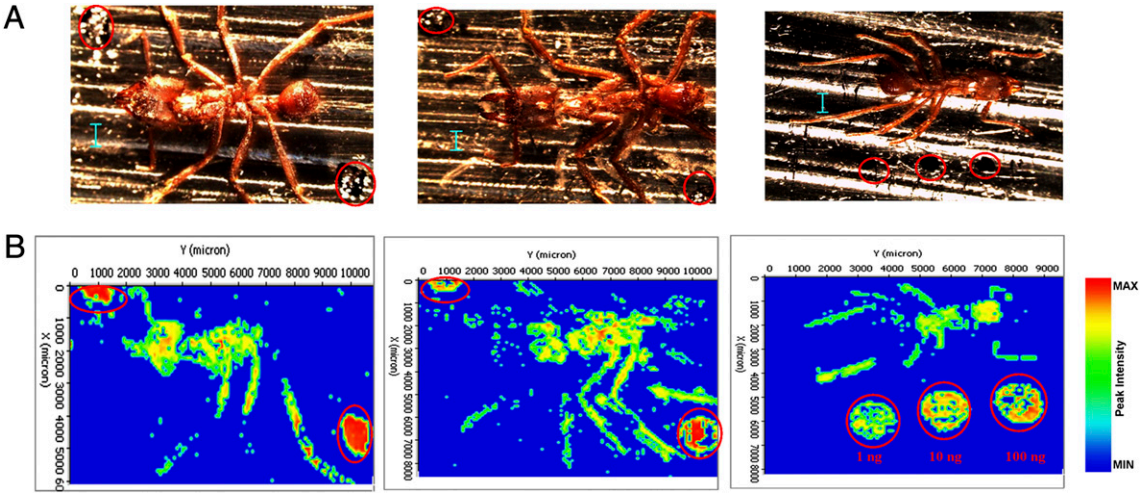

Fig. 2. (*A*) Microscopic pictures of *A. echinatior* workers mounted onto MALDI plates. Beside the ants are valinomycin spots as reference to estimate the amount of valinomyin (**3**) on the integument of leaf-cutting ants (red circles). (Scale bars in the microscope images, 1 mm.) (*B*) MALDI images reflect the distribution of valinomycin (**3**) ($[M+K]^+$ *m/z* = 1,149) on the integument of the *A. echinatior* workers shown in *A*. The color code of the heat maps is logarithmic and corresponds to different concentrations of valinomycin (**3**). During the MALDI analysis, the abdomen of all ants fell off, and therefore, there are no data of the valinomycin distribution on the abdomen available.

of the respiratory chain and thus induce apoptosis (30, 35). Thus, antimycins A_1–A_4 (**4–7**) inhibit *Candida albicans*, *Mucor mucedo*, and *Aspergillus niger*; however, they are not particularly active against *Fusarium* (36), which is a pathogen in the ants' nests. In addition, antimycins (**4–7**) were found to inhibit the growth of several bacteria (37) and yeasts (35). Antimycins A_1–A_4 (**4–7**) inhibited the growth of *E. weberi* (Table 1) and the detrimental black yeast *P. fastigiata* (32) in our agar diffusion assays against pathogens of the leaf-cutting ants. In addition, we observed a strongly antagonistic effect of antimycins A_1–A_4 (**4–7**) against the mutualistic fungus *L. gongylophorus*. This provides a chemical explanation for recent observations, namely that the microbial symbionts of leaf-cutting ants have negative as well as positive effects on the ant colony (18, 23). In the case of fungal infection, the cost of inhibiting growth of the garden fungus *L. gongylophorus* may be outweighed by the benefit of preventing the spread of infection in the nest.

Valinomycin (**3**) (28) and structural variants (29) are ionophores that selectively bind potassium ions, leading to the disintegration of the cellular membrane potential and thus, destroying cells (38). However, in the agar diffusion assays, valinomycins did not inhibit the fungi tested, including the symbiotic garden fungus (Table 1). Nevertheless, valinomycin (**3**) is known to inhibit the hyphal growth of *C. albicans* (39) as well as various phytopathogens (40, 41). The ability to defend against phytopathogens is potentially relevant, because leaf-cutting ants may import these pathogens with the leaf material that they collect to feed *L. gongylophorus* (5). Additionally, valinomycin (**3**) is active against insects, nematodes, and mites (42).

Actinomycins have been identified as highly active antibiotics (43), but on account of their high toxicity, actinomycins are used exclusively in cancer therapy (44). Because actinomycins strongly inhibit the growth of competing microorganisms, including other *Streptomyces* and *Pseudonocardia* symbionts of leaf-cutting ants (Table S1), they obviously help *Streptomyces* sp. Av25_2 compete with neighboring microorganisms. Thus, with actinomycins identified as being synthesized by a *Streptomyces* strain isolated from leaf-cutting ants, we provide the chemical basis for the observation of Sen et al. (18), namely that ant-associated microorganisms play different roles in this ecosystem, ranging from mutualistic to detrimental interactions. Competition between their different microbial symbionts may, at first glance, be disadvantageous for the leaf-cutting ants; nevertheless, it may provide a pathway for the selection of potent defenders by inducing an evolutionary arms race between them.

Because antimycins A_1–A_4 (**4–7**), actinomycins, and valinomycins as well as the previously identified candicidin macrolides (11) are likely to occur in concert in the leaf-cutting ants' nest, we investigated the inhibitory properties of different compound mixtures. We found, indeed, that as mixtures, these antibiotics inhibited *E. weberi* growth more efficiently than the single compounds. Amounts close to or below the minimal inhibitory concentration of each compound together caused clear inhibition zones in the agar diffusion assay (Figs. S7 and S8). Such synergistic effects between valinomycin (**3**) or actinomycin D (**1**) with polyene macrolides have been observed previously in pharmacological studies (33, 34). Now, we show that chemical diversity, particularly, the interplay of bioactive molecules, is an important factor in the protection of the leaf-cutting ants' nests against infections.

Using MALDI imaging, we were able to identify and monitor the local distribution of an antibiotic, valinomycin (**3**), from microbial symbionts of leaf-cutting ants directly on the integument (Fig. 2). On the cuticle of *A. echinatior* workers, patches with high amounts of valinomycin (**3**) (e.g., at joints of the legs) were detected. Considering the weight of the *A. echinatior* workers used for the MALDI imaging (~6 mg), several nanograms of valinomycin (**3**) and in some patches, several tenths of nanograms are likely to be sufficient to fight against susceptible organisms. Until now, it has only been possible to directly detect antibiotics of microbial symbionts from insects on the cocoon of beewolf larvae (45). The presence of valinomycin (**3**) on the ants' bodies suggests that it may play an important role in protecting individual workers, probably not only against microbial pathogens but also against parasites (e.g., mites) (1, 42). In addition, we found actinomycins (**1, 2**) and valinomycin (**3**) in the waste of *A. niger* and *A. echinatior*, which shows again that *Streptomyces* sp. play an essential role (11, 16) in the environment of leaf-cutting ants. The high variability in the quantities of valinomycin (**3**) in the waste of *A. niger* provides molecular proof that the antibiotics of the microbial symbionts of leaf-cutting ants can be highly localized (46). Therefore, it seems possible that the production of antibiotics may be regulated by the producing microorganisms or even the leaf-cutting ants (46). The direct detection of antibiotics in natural environments is often problematic, because these compounds can be active in very low concentrations and their occurrence can vary both locally and

temporally (47). The limited quantities of fungus garden and waste samples available to us may explain why we found only valinomycins and in some cases, actinomycins (**1, 2**) in our extracts.

Leaf-cutting ants likely benefit from the rich diversity of antimicrobial and antifungal secondary metabolites from their microbial symbionts, because combined, these antibiotics can have strong synergistic effects against possible threats; however, these compounds can also have detrimental side effects. The diversity of natural products plays a driving role in shaping the ecosystems of leaf-cutting ants. Only by revealing their chemical nature we can begin to understand fully the complex interactions between multiorganismic partners. The combined approach of phylogenetic analysis with chemical analytics presented here can significantly speed up the identification of the diverse bioactive natural products that orchestrate the multiple interactions in complex biological systems.

Materials and Methods

Fungal and Microbial Cultures. *E. weberi* CBS 110660 was obtained from the Centralbureau voor Schimmelcultures in Utrecht, The Netherlands. *L. gongylophorus* was an isolate from the fungus garden of *Atta colombica* (laboratory colony collected in Gamboa, Panama, in 2004). *F. decemcellulare* was from the Phytopathology Department of the Friedrich Schiller University Jena, and *B. bassiana* FSU 5084 was obtained from the Pilzreferenzzentrum of the Friedrich Schiller University Jena (Jena, Germany). *T. harzianum* DSM 63059, *P. fastigiata* DSM 2692, *M. anisopliae* DSM 1490, *C. militaris* DSM 1153, *B. subtilis* DSM 10, and *S. racemosum* DSM 859 originated from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The *Streptomyces* and *Pseudonocardia* strains used for bioassays were isolates from *A. volcanus*, *A. octospinosus*, and *A. echinatior* leaf-cutting ants (11). All strains were maintained on soy flour medium (SFM) agar plates (20 g soy flour, 20 g mannitol, 15 g agar, 1 L ddH_2O) (48).

Leaf-Cutting Ants and Fungus Garden Samples. Leaf-cutting ants and fungus garden samples from *A. volcanus*, *A. octospinosus*, and *A. echinatior* colonies were collected and identified in 2007 in Gamboa (Panama) by H.H. In addition, microorganisms were isolated from the fungus garden of an established laboratory colony of *A. echinatior* collected in 2002 by H.H. in Panama. Fungus garden samples and waste samples were collected from *A. echinatior* and *A. niger* laboratory colonies (collected in Brazil in 1999 and maintained as laboratory colonies by R.W.).

Cultivation of Microorganisms. *Streptomyces* from *Acromyrmex* leaf-cutting ants were isolated and determined by Haeder et al. (11). All *Streptomyces* were maintained on SFM agar plates (48). To obtain methanol extracts, the microorganisms were grown in 20 mL test tubes fitted with springs for aeration containing 6 mL liquid SFM. The cultures were incubated at 28 °C on an orbital shaker (220 rpm, Infors Multitron II MT25) for 6 d; 2 mL culture were lyophilized and redissolved in 1 mL methanol. For larger-scale isolation of secondary metabolites, 200 mL liquid SFM were filled into 500-mL Erlenmeyer flasks fitted with springs for aeration. The flasks were inoculated with a *Streptomyces* spore suspension. The cultures were grown in an orbital shaker (220 rpm at 28 °C, Infors Multitron II MT25) for 6 d.

Secondary Metabolite Screening Inspired by Phylogenetic Data. 16*S* rDNA data analysis was performed for *Streptomyces* symbionts obtained previously from three *Acromyrmex* leaf-cutting ant species (11). Highly similar sequences using blast algorithm were identified using the Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) (49) and NCBI (http://www.ncbi.nlm.nih.gov/nuccore) databases. The hits for well-studied, closely-related microorganisms with similarities higher than >98.5% were screened for their antimicrobial and antifungal secondary metabolites using SciFinder from the CAS. The construction of phylogenetic trees was performed with the program MEGA version 4 using the neighbor-joining method (bootstrap value, $n = 1{,}000$) (50).

LC-ESI-MS Analysis of Extracts from Microbial Symbionts. Fifteen microliters of extract were injected into a Dionex Ultimate 3000 HPLC system fitted with a Phenomenex Kinetex C18 column (2.6 μm, 100 Å, 150 × 2.1 mm). Either a Thermo Fisher LTQ or an LTQ Orbitrap with an ESI ion source served as an MS detector. HPLC conditions: 3 min in 100% A, 27 min to 100% B, 10 min in 100% B (A, H_2O 0.5% AcOH; B, MeCN 0.5% AcOH; flow rate = 0.20 mL/min). Compounds were identified by their retention times and HR-ESI-MS/MS spectra compared with commercially available standards.

Actinomycins (1, 2) from *Streptomyces* sp. Av25_2. Based on the 16*S* rDNA similarity of *Streptomyces* sp. Av25_2 to *S. parvus* (99.57%), which is a known producer of actinomycin C (25), a methanol extract from a *Streptomyces* sp. Av25_2 culture (6 mL) was screened for actinomycin production by LC-MS. Retention time of actinomycin D (**1**): 24.8 min (HR-ESI-MS: $[M+H]^+$ measured: 1,255.6353, calculated: 1,255.6363 $C_{62}H_{87}N_{12}O_{16}$); retention time of actinomycin X_2 (**2**): 25.2 min (ESI-HR-MS: $[M+H]^+$ measured: 1,269.6143, calculated: 1,269.6156 $C_{62}H_{85}N_{12}O_{17}$).

Methanol extracts of all microbial symbionts (11) from *Acromyrmex* leaf-cutting ants were screened for actinomycin production using LC-MS.

Valinomycin (3) and Valinomycin Derivatives from *Streptomyces* Symbionts of *Acromyrmex*. *Streptomyces* sp. Av25_3 exhibited high similarity to known valinomycin producers *S. tsusimaensis* (99.21%) and *S. anulatus* (98.84%). Methanol extracts of *Streptomyces* sp. Av25_3 were screened by LC-MS and compared with the retention time of an authentic standard of valinomycin.

Retention times of valinomycin (**3**) and valinomycin derivatives (29): valinomycin-28 amu: 41.5 min (ESI-HR-MS: $[M+NH_4]^+$ measured: 1,100.6338, calculated: 1,100.6342 $C_{52}H_{90}N_7O_{18}$; ESI-HR-MS: $[M+K]^+$ measured: 1,121.5621, calculated: 1,121.5630 $C_{52}H_{86}N_6O_{18}K$); valinomycin-14 amu: 43.6 min (ESI-HR-MS: $[M+NH_4]^+$ measured: 1,114.6499, calculated: 1,114.6493 $C_{53}H_{92}N_7O_{18}$; ESI-HR-MS: $[M+K]^+$ measured: 1,135.5776, calculated: 1,135.5787 $C_{53}H_{88}N_6O_{18}K$); valinomycin (**3**): 46.3 min (ESI-HR-MS: $[M+NH_4]^+$ measured: 1,128.6658, calculated: 1,128.6650 $C_{54}H_{94}N_7O_{18}$; ESI-HR-MS: $[M+K]^+$ measured: 1,149.5936, calculated: 1,149.5949 $C_{54}H_{90}N_6O_{18}K$); valinomycin+14 amu: 49.7 min (ESI-HR-MS: $[M+NH_4]^+$ measured: 1,142.6808, calculated: 1,142.6806 $C_{55}H_{94}N_7O_{18}$; ESI-HR-MS: $[M+K]^+$ measured: 1,163.6087, calculated: 1,163.6100 $C_{55}H_{90}N_6O_{18}K$).

Methanol extracts of all microbial symbionts (11) from *Acromyrmex* leaf-cutting ants were screened for valinomycin production using LC-MS.

Antimycins A_1–A_4 (4–7) from *Streptomyces* Symbionts of *Acromyrmex*. *S. albidoflavus*, a producer of the antifungal antimycins such as antimycin A_{18} (31), exhibited high similarity to several *Streptomyces* symbionts from the leaf-cutting ants: *Streptomyces* sp. Ae32_2 (*S. albidoflavus*, AJ002090.1; 100.00%), *Streptomyces* sp. Ao10 (*S. albidoflavus*, DQ855477.1; 99.72%), *Streptomyces* sp. Av28_2 (*S. albidoflavus* str. UST040711-291, FJ591130.1; 98.23%), *Streptomyces* sp. Av28_3 (*S. albidoflavus*, AJ002090.1; 99.07%), *Streptomyces* sp. Av25_1 (*S. albidoflavus*, AJ002090.1; 97.78%), and *Streptomyces* sp. Av26_5 (*S. albidoflavus*, AJ002090.1; 99.93%).

Retention time of antimycin A_1 (**4**): 29.0 min (ESI-HR-MS: $[M+H]^+$ measured: 549.2805, calculated: 549.2812 $C_{28}H_{41}O_9N_2$); retention time antimycin A_2 (**5**): 28.0 min (ESI-HR-MS: $[M+H]^+$ measured: 535.2651, calculated: 535.2656 $C_{27}H_{39}O_9N_2$); retention time antimycin A_3 (**6**): 27.0 min (ESI-HR-MS: $[M+H]^+$ measured: 521.2493, calculated: 521.2500 $C_{26}H_{37}O_9N_2$); retention time antimycin A_4 (**7**): 26.0 min (ESI-HR-MS: $[M+H]^+$ measured: 507.2342, calculated: 507.2343 $C_{25}H_{35}O_9N_2$).

Methanol extracts of all microbial symbionts (11) from *Acromyrmex* leaf-cutting ants were screened for antimycin A_1–A_4 (**4–7**) production using LC-MS.

Antimicrobial and Antifungal Properties of Antimycins A_1–A_4 (4–7), Actinomycins (1, 2), Valinomycin (3), and Valinomycin Derivatives. *E. weberi*, *L. gongylophorus*, *F. decemcellulare*, *B. bassiana*, *T. harzianum*, *P. fastigiata*, *M. anisopliae*, *C. militaris*, *S. racemosum*, *B. subtilis*, *Streptomyces* sp. Av25_2, *Streptomyces* sp. Av25_4, *Streptomyces* sp. Ao10, *Pseudonocardia* sp. Ao1, and *Pseudonocardia* sp. Av30 were used as test organisms in the agar diffusion assay against antimycins A_1–A_4 (**4–7**) (5.8–580.0 nmol), actinomycins (**1, 2**) (0.4–240.0 nmol), and valinomycins (1.4–270.0 nmol). In addition, the performance of *E. weberi* in the presence of compound mixtures was investigated.

For the bioassays, 100 μL mycelium or spore suspensions (~5 mg wet weight/mL in water) of the test organisms were spread onto SFM plates (5.5 cm diameter, 10 mL medium). A 6-mm hole was cut in the middle of the plate to apply 50 μL test solutions or an appropriate solvent control (MeOH). The inhibition zones were monitored after samples were incubated for 4–16 d at 28 °C. All assays that showed inhibition zones were performed at least in triplicate and compared with identically prepared solvent controls. The results of the assays are presented in Table 1, Table S2, and Figs. S2–S8.

Detection of Antibiotic and Antifungal Compounds in the Fungus Garden and Waste. Waste and fungus garden samples from *A. echinatior* and *A. niger* were analyzed. The samples (0.58–31.81 g) were extracted with ethyl acetate (10–300 mL) by sonification and stirring for 2 h. After filtration, the filtrate was concentrated in vacuo. The residue was resuspended in 200 μL, 500 μL, or 2,500 μL methanol and analyzed by LC-MS. The measurements were compared with standards of actinomycin D (**1**) and X_2 (**2**), antimycins A_1–A_4

(**4–7**), valinomycin (**3**), and valinomycin derivatives. The total amounts of valinomycins and actinomycins in the extracts were estimated by comparison of the respective peak areas to an external calibration curve.

MALDI Imaging of *A. echinatior* Workers. *A. echinatior* workers were mounted onto the MALDI plate with the help of a double-sided adhesive tape and sprayed with matrix solution of α-cyano-4-hydroxycinnamic acid (51) [7 mg/mL in MeCN:water (80:20, v:v) and 0.2% trifluoroacetic acid] by an automatic sprayer (Bruker ImagePrep). Microscopic pictures (magnification = 1–1.25×) were taken using a Leica S8 APO Greenough stereo microscope equipped with a Schott KL 1500 compact halogen cold light source. The images were captured by a digital camera and were processed using the Leica Application Suite LAS EZ ver. 1.6.0. All images were rescaled to the same scale ratio to make parity among all of the different images. MALDI imaging experiments of antibiotics on the cuticle of *A. echinatior* workers were performed by an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher) coupled to a MALDI source to provide spectra and images. The spectrometer was operated in positive-selected ion monitoring mode (mass range = 1,060–1,160) with nominal mass resolving power of 60,000 at *m/z* 400 and a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2-ppm deviation using the internal calibration standard α-cyano-4-hydroxycinnamic acid (CHCA):*m/z* = 379.095. The laser was set at power 20 μJ, with raster plate motion and raster step size of 100 μm; three microscans per step are used for the analysis. All data were processed by Thermo ImageQuest 1.0.1 software.

To estimate the amount of valinomycin (**3**) ($[M+K]^+$ *m/z* = 1,149.602) on the integument of leaf-cutting ants, a dilution series of valinomycin (**3**) (1, 10, and 100 ng) was measured under identical conditions as the ant samples.

ACKNOWLEDGMENTS. We thank the Smithsonian Tropical Research Institute (STRI) for providing logistic help and the Autoridad Nacional del Ambiente y el Mar (ANAM) for permission to sample ant colonies in Panama and for issuing export permits. We are indebted to Dr. Kusari for performing the stereomicroscope imaging and Emily Wheeler for editorial assistance. D.S. thanks Professor Dr. Boland for his generous support, the Deutsche Forschungsgemeinschaft for an Emmy Noether Fellowship (SP 1106/3-1), and the Jena School for Microbial Communication (JSMC) for financial support. Further funding by the Verband der Chemischen Industrie and the Max Planck Society is gratefully acknowledged.

1. Hölldobler B, Wilson EO (1990) *The Ants* (Springer, Berlin).
2. Seifert KA, Samson RA, Chapela IH (1995) *Escovopsis aspergilloides*, a rediscovered hyphomycete from leaf-cutting ant nests. *Mycologia* 87:407–413.
3. Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. *Proc Natl Acad Sci USA* 96:7998–8002.
4. Rodrigues A, et al. (2005) Variability of non-mutualistic filamentous fungi associated with *Atta sexdens rubropilosa* nests. *Folia Microbiol (Praha)* 50:421–425.
5. Fisher PJ, Stradling DJ, Sutton BC, Petrini LE (1996) Microfungi in the fungus gardens of the leaf-cutting ant *Atta cephalotes*: A preliminary study. *Mycol Res* 100:541–546.
6. Bot ANM, Currie CR, Hart AG, Boomsma J (2001) Waste management in leaf-cutting ants. *Ethol Ecol Evol* 13:225–237.
7. Schildknecht H, Koob K (1971) Myrmicacin, the first insect herbicide. *Angew Chem Int Ed Engl* 10:124–125.
8. Bot ANM, Ortius-Lechner D, Finster K, Maile R, Boomsma JJ (2002) Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Soc* 49:363–370.
9. Fernandez-Marin H, Zimmerman JK, Rehner SA, Wcislo WT (2006) Active use of the metapleural glands by ants in controlling fungal infection. *Proc R Soc Lond B Biol Sci* 273:1689–1695.
10. Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398:701–704.
11. Haeder S, Wirth R, Herz H, Spiteller D (2009) Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proc Natl Acad Sci USA* 106:4742–4746.
12. Oh DC, Poulsen M, Currie CR, Clardy J (2009) Dentigerumycin: A bacterial mediator of an ant-fungus symbiosis. *Nat Chem Biol* 5:391–393.
13. Barke J, et al. (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. *BMC Biol* 8:109.
14. Currie CR, et al. (2003) Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science* 299:386–388.
15. Santos AV, Dillon RJ, Dillon VM, Reynolds SE, Samuels RI (2004) Ocurrence of the antibiotic producing bacterium *Burkholderia* sp. in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. *FEMS Microbiol Lett* 239:319–323.
16. Kost C, et al. (2007) Non-specific association between filamentous bacteria and fungus-growing ants. *Naturwissenschaften* 94:821–828.
17. Pinto-Tomás AA, et al. (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science* 326:1120–1123.
18. Sen R, et al. (2009) Generalized antifungal activity and 454-screening of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants. *Proc Natl Acad Sci USA* 106:17805–17810.
19. Jensen PR, Williams PG, Oh DC, Zeigler L, Fenical W (2007) Species-specific secondary metabolite production in marine actinomycetes of the genus Salinispora. *Appl Environ Microbiol* 73:1146–1152.
20. Matter AM, Hoot SB, Anderson PD, Neves SS, Cheng YQ (2009) Valinomycin biosynthetic gene cluster in *Streptomyces*: Conservation, ecology and evolution. *PLoS ONE* 4:e7194.
21. Jørgensen H, et al. (2009) Candicidin biosynthesis gene cluster is widely distributed among *Streptomyces* spp. isolated from the sediments and the neuston layer of the Trondheim fjord, Norway. *Appl Environ Microbiol* 75:3296–3303.
22. Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Can J Microbiol* 51:441–446.
23. Mueller UG, Dash D, Rabeling C, Rodrigues A (2008) Coevolution between attine ants and actinomycete bacteria: A reevaluation. *Evolution* 62:2894–2912.
24. Zucchi TD, Guidolin AS, Cônsoli FL (2010) Isolation and characterization of actinobacteria ectosymbionts from *Acromyrmex subterraneus brunneus* (Hymenoptera, Formicidae). *Microbiol Res* 166:68–76.
25. Mordarski M, Skurska H, Morawiecki A (1958) Antibacterial properties of *Streptomyces* V. Identification of a *Streptomyces parvus* antibiotic. *Arch Immunologii I Terapii Doswiadczalnej* 6:367–373.
26. Bullock E, Johnson AW (1957) Actinomycin 5. The structure of actinomycin D. *J Chem Soc* 3280–3285.
27. Brockmann H, Manegold JH (1960) Actinomycine. 23. Antibiotica aus Actinomyceten. 45. Überführung von Actinomycin X_2 in die Actinomycine C_1, X_0-β und X_0-δ. *Chem Ber* 93:2971–2982.
28. Shemyakin MM, Aldanova NA, Vinogradova EI, Feigina MY (1963) The structure and total synthesis of valinomycin. *Tetrahedron Lett* 1921–1925.
29. Pitchayawasin-Thapphasaraphong S, Isobe M (2006) Molecular diversity of valinomycin, a 36-membered cyclic depsipeptide, which was detected by means of HPLC-Q-TOF-MS on hydrolysate dipeptides. ITE Letters on Batteries. *New Technol Med* 7:465–473.
30. van Tamelen E, Strong FM, Loomans ME, Dickie JP, Dewey RS (1961) Chemistry of antimycin A. 10. Structure of antimycins. *J Am Chem Soc* 83:1639–1646.
31. Yan LL, et al. (2010) Antimycin A18 produced by an endophytic Streptomyces albidoflavus isolated from a mangrove plant. *J Antibiot (Tokyo)* 63:259–261.
32. Little AEF, Currie CR (2007) Symbiotic complexity: Discovery of a fifth symbiont in the attine ant-microbe symbiosis. *Biol Lett* 3:501–504.
33. Kwan CN, Medoff G, Kobayashi GS, Schlessinger D, Raskas HJ (1972) Potentiation of the antifungal effects of antibiotics by amphotericin B. *Antimicrob Agents Chemother* 2: 61–65.
34. Bolard J (1986) How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim Biophys Acta* 864:257–304.
35. Kluepfel D, Sehgal SN, Vézina C (1970) Antimycin A components. I. Isolation and biological activity. *J Antibiot (Tokyo)* 23:75–80.
36. Ueki M, et al. (1997) UK-3A, a novel antifungal antibiotic from Streptomyces sp. 517-02: Fermentation, isolation, structural elucidation and biological properties. *J Antibiot (Tokyo)* 50:551–555.
37. Marquis RE (1965) Nature of bactericidal action of antimycin A for *Bacillus megaterium*. *J Bacteriol* 89:1453–1459.
38. Pinkerton M, Steinrauf LK, Dawkins P (1969) The molecular structure and some transport properties of valinomycin. *Biochem Biophys Res Commun* 35:512–518.
39. Watanabe H, Azuma M, Igarashi K, Ooshima H (2005) Valinomycin affects the morphology of *Candida albicans*. *J Antibiot (Tokyo)* 58:753–758.
40. Lim TH, et al. (2007) Antifungal activity of valinomycin, a cyclodepsipeptide from *Streptomyces padanus* TH-04. *Nat Prod Sci* 13:144–147.
41. Park CN, Lee JM, Lee D, Kim BS (2008) Antifungal activity of valinomycin, a peptide antibiotic produced by *Streptomyces* sp. Strain M10 antagonistic to *Botrytis cinerea*. *J Microbiol Biotechnol* 18:880–884.
42. Heisey RM, et al. (1988) Production of valinomycin, an insecticidal antibiotic, by *Streptomyces griseus* var. *flexipertum* var. *nov*. *J Agric Food Chem* 36:1283–1286.
43. Waksman SA, Tishler M (1942) The chemical nature of actinomycin, an antimicrobial substance produced by *Actinomyces antibioticus*. *J Biol Chem* 142:519–528.
44. Moore GE, Dipaolo JA, Kondo T (1958) The chemotherapeutic effects and complications of actinomycin D in patients with advanced cancer. *Cancer* 11:1204–1214.
45. Kroiss J, et al. (2010) Symbiotic Streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nat Chem Biol* 6:261–263.
46. Fernandez-Marin H, Zimmerman JK, Nash DR, Boomsma JJ, Wcislo WT (2009) Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. *Proc R Soc Lond B Biol Sci* 276:2263–2269.
47. Anukool U, Gaze WH, Wellington EMH (2004) In situ monitoring of streptothricin production by *Streptomyces rochei* F20 in soil and rhizosphere. *Appl Environ Microbiol* 70:5222–5228.
48. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) *Practical Streptomyces Genetics* (Crowes, Norwich, United Kingdom).
49. DeSantis TZ, et al. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72:5069–5072.
50. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599.
51. Beavis RC, Chaudhary T, Chait BT (1992) α-Cyano-4-hydroxycinnamic acid as a matrix for matrix-assisted laser desorption mass-spectrometry. *Org Mass Spectrom* 27: 156–158.